Immunity, Vol. 8, 761–769, June, 1998, Copyright ©1998 by Cell Press

In Vitro Generation of Lymphohematopoietic Cells from Endothelial Cells Purified from Murine Embryos

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Summary

We have investigated the lymphohematopoietic potentials of endothelial cells (EC) and hematopoietic cells (HPC) sorted from embryos. Expression of VE-cadherin, CD45, and Ter119 was used to distinguish EC (VE-cadherin$^+$CD45$^-$Ter119$^-$) from HPC (VE-cadherin$^-$CD45$^+$). Thus defined, EC population takes up acetylated LDL and coexpresses CD31, Flk1, and CD34. In E9.5 embryos, EC from yolk sac (YS) and the embryo proper generate blood cells, including lymphocytes. Thus, lymphohematopoietic EC do exist in the embryo, and they are generated both in YS and the embryo proper. On the other hand, HPC with lymphopoietic potency appear first in the embryo proper. These findings implicate involvement of multiple environmental cues for acquiring lymphopoietic competency during differentiation of HPC.

Introduction

Controversy regarding the first site of the generation of hemopoietic cells (HPC) with lymphohemogenic potency has raged for three decades, resurfacing whenever innovations have been introduced in measuring the potency of HPC (Moore and Owen, 1967; Johnson and Moore, 1975; Weissman et al., 1978; Ogawa et al., 1988; Muller et al., 1994; Cumano et al., 1996; Medvinsky and Dzierzak, 1996; Dieterlen-Lievre et al., 1997; Yoder et al., 1997a, 1997b). It is surprising that conflicting notions have survived this long, considering that the issue is a simple one: whether or not HPC in the yolk sac (YS) display the cell autonomous potential to give rise to the definitive hematopoietic stem cells. In this regard, experiments with positive results emphasize the importance of experimental conditions for generating lymphocytes, whereas those with negative results stress the potential interchange of stem cells by the embryonic circulation. In neither case, however, has the possibility that endothelial cells (EC) bear the potential to give rise to lymphohemopoietic lineages been considered. Therefore, all previous studies prepared cell suspensions from YS either by mechanical dissociation or collagenase treatment, which are not sufficient to dissociate cells from the organized EC layer. However, histological findings that HPC appeared to bud from the luminal wall of the developing vascular system (Smith and Glomski, 1982; Tavian et al., 1996) suggested that some EC, even after being integrated in the EC layer, can give rise to HPC. If such hematopoietic EC exist, the analysis of the lymphopoietic potential of various tissues should be extended to EC. Histological observation, however, is not sufficient to prove the presence of hematopoietic EC, because it is difficult to distinguish whether HPC bud from EC or circulating HPC somehow adhere to EC.

The first aim of this study is to investigate whether or not hematopoietic EC exist in the earliest hematopoietic tissues, such as YS and the aorta-gonado-mesonephros region (AGM). If they do, then the next aim is to investigate the lymphopoietic potential of EC in the YS and the embryo proper. In order to measure hematopoietic potential of EC, however, it is essential to purify EC with surface markers distinguishing EC from HPC and also from earlier pluripotent mesoderm. According to previous findings that VE-cadherin (VE-cad) is expressed exclusively in EC and plays an essential role in maintaining the integrity of the EC layer (Ayalon et al., 1994; Lampugnani et al., 1995; Matsuyoshi et al., 1997; Vittet et al., 1997), we sought to purify EC by surface staining with anti-VE-cad monoclonal antibodies (mAb).

In this report, we show that VE-cad is indeed expressed specifically in EC. VE-cad$^+$CD45$^-$Ter119$^-$ cells sorted by fluorescence-activated cell sorter (FACS) from either YS or the caudal half of the embryo proper can give rise to lymphohematopoietic cells, providing the first evidence for the presence of lymphohematopoietic EC. Surprisingly, the lymphopoietic potential of embryonic day (E) 9.5 YS is enriched in the VE-cad$^+$ fraction rather than the CD45$^+$ or Ter119$^+$ HPC fraction. Our results indicate that the lymphohematopoietic potentials of YS and the caudal half of the embryo proper, including the AGM region, differ at the HPC level, while they are basically the same at the EC level. Hence, this study may help to settle the 30-year controversy on the lymphohematopoietic potency of YS cells.

Results

VE-Cadherin Expression in Early Embryos

A previous study indicated that VE-cadherin is expressed exclusively in EC (Breier et al., 1996). To assess this notion using our own mAb, E7.5 and 9.5 embryos were immunostained with anti-VE-cadherin mAb VECD1 (Figure 1). In the early head streak stage (E7.5), when VE-cad was reported to be first expressed (Breier et al., 1996), VE-cad$^+$ cells appeared to constitute a small part of the Flk1$^+$ cells that are distributed over the extraembryonic and lateral regions of embryos (Figures 1A and 1B). VE-cad was expressed in the cells that had already been organized to form a vascular plexus, while Flk1$^+$ cells were still distributed diffusely with a dot-like pattern. This pattern suggests that VE-cad expression coincides well with the formation of vascular structure, while

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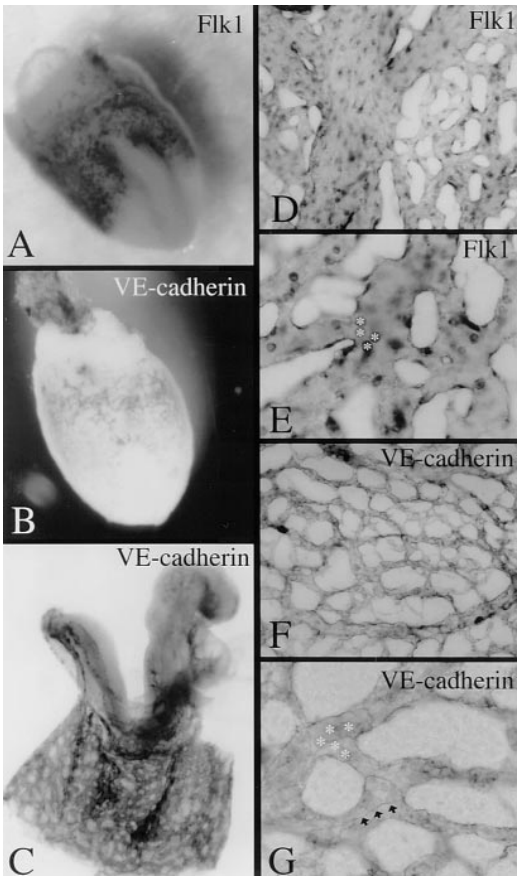

Figure 1. Immunolocalization of VE-Cad in Embryos

Early head fold stage embryos were dissected and whole-mount immunostained either with anti-VE-cad (A) or anti-Flk1 (B). As compared with diffuse dot-like expression of Flk in the extraembryonic and lateral plate regions, VE-cad$^+$ cells are already organized in the plexus-like structure. Colocalization of VE-cad to vascular structures becomes conspicuous in the YS of E8.5 embryos (C). YS of E9.5 embryos were stained either with anti-Flk1 (D and E) or anti-VE-cad mAb (F and G). At this stage, Flk1 colocalize largely to vascular structures, though many round cells in the luminal space (E, indicated by white asterisks) were also positive. In contrast, those round cells were negative for VE-cad expression (G, indicated by white asterisks). Thus, VE-cad is expressed exclusively in the EC, particularly in the cell–cell junctions of EC (arrows in G).

Flk1 marks both mesoderm and EC in the gastrulating embryo. Indeed, previous studies demonstrated that Flk1 is expressed in the nascent proximal lateral mesoderm of E7.0 embryos before expression of VE-cad (Kataoka et al., 1997; Nishikawa et al., 1998).

Whole-mount immunostaining of E8.5 embryo (Figure 1C) and YS of E9.5 embryos (Figures 1F and 1C) with anti-VE-cad mAb displayed clearly that VE-cad expression localized exclusively in the vascular system and that it was concentrated at the adherence junctions of EC (arrows in Figure 1G). At this stage, Flk1 expression also localized to the vascular endothelial cells (Figures 1D and 1E), though some round cells in the vascular lumens (indicated by asterisk in Figure 1E) were Flk1$^+$. In contrast, round HPC present within the luminal space (indicated by asterisk in Figure 1G) were VE-cad$^-$. No staining was found in the extraluminal space. These results agree with the conclusion of a previous study that VE-cad is expressed exclusively in EC (Breier et al., 1996), while Flk1$^+$ cells include mesoderm, EC, and some hematopoietic cells. Another marker, CD31 (platelet endothelial cell adhesion molecule), that has often been used for marking EC, was expressed also in non-mesoderm-derived cells (Lampugnani et al., 1993; our unpublished data).

Surface Phenotypes of VE-Cad$^+$ Cells

Since whole-mount immunostaining may not be sufficient to detect a small number of VE-cad$^+$ cells that coexpress HPC markers, we next analyzed expression of other surface markers on VE-cad$^+$ cells using FACS. Single cell suspensions were prepared from various stages of embryos (Figure 2) by sequential treatment with dispase and EDTA/EGTA. Without this treatment, the VE-cad$^+$ EC fraction could not be recovered, indicating that most VE-cad$^+$ cells are indeed organized in the EC layer by adherence junction.

CD45$^+$ cells were detected earlier in YS than the embryo proper (in this study the embryo proper stands for the caudal half of the embryo lower than heart level), implicating YS as the first site of HPC generation. CD34 expression was detected in both CD45$^+$ and VE-cad$^+$ populations, and virtually all CD34$^+$ cells present in embryos younger than E9.5 coexpressed c-Kit as well. Upon generation of differentiated HPC, c-Kit$^-$ CD34$^+$ appeared. In contrast to CD34 expression in the CD45$^+$ population, VE-cad-expression and CD45-expression are mutually exclusive before E9.5 (Figure 2), though some Mac1$^+$ monocytes appearing later than E10.5 expressed VE-cad. In order to confirm EC lineage of VE-cad$^+$ cells, cells dissociated from YS and the caudal half of E9.5 embryos were pooled, first incubated with DiI-acetylated LDL, and subsequently stained by APC-anti-VE-cad and fluorescein isothiocyanate (FITC)-labeled anti-CD31, anti-Flk1, or anti-CD34 mAb. As shown in Figure 3, almost all VE-cad$^+$ cells shifted to the right with these mAb or incubation with DiI-acetylated LDL, indicating that virtually all VE-cad$^+$ cells coexpress CD31, Flk1, and CD34 and take up acetylated LDL. In these criteria, thus, VE-cad$^+$ cells represent functional EC.

Colony Formation of VE-Cad$^+$ Cells in Response to Hematopoietic Growth Factors

In order to investigate whether or not hematopoietic EC exists, cells dissociated from YS and the caudal half of E9.5 embryos were pooled, incubated with DiI-acetylated LDL, and subsequently stained with APC-labeled anti-VE-cad and FITC-labeled anti-CD31. Triple-positive cells were sorted and subjected to colony assay in type I collagen gel containing SCF, interleukin-3 (IL-3), G-CSF, and erythropoietin. While no colony was generated in the culture without growth factors, 277 colonies were generated in cultures containing 2500 sorted cells and the growth factor mixture. Thus, more than 10% of VE-cad$^+$ CD31$^+$ cells incorporating acetylated LDL can form colonies in response to the growth factor mixture. Both compact and diffuse colonies were found (Figures 4A

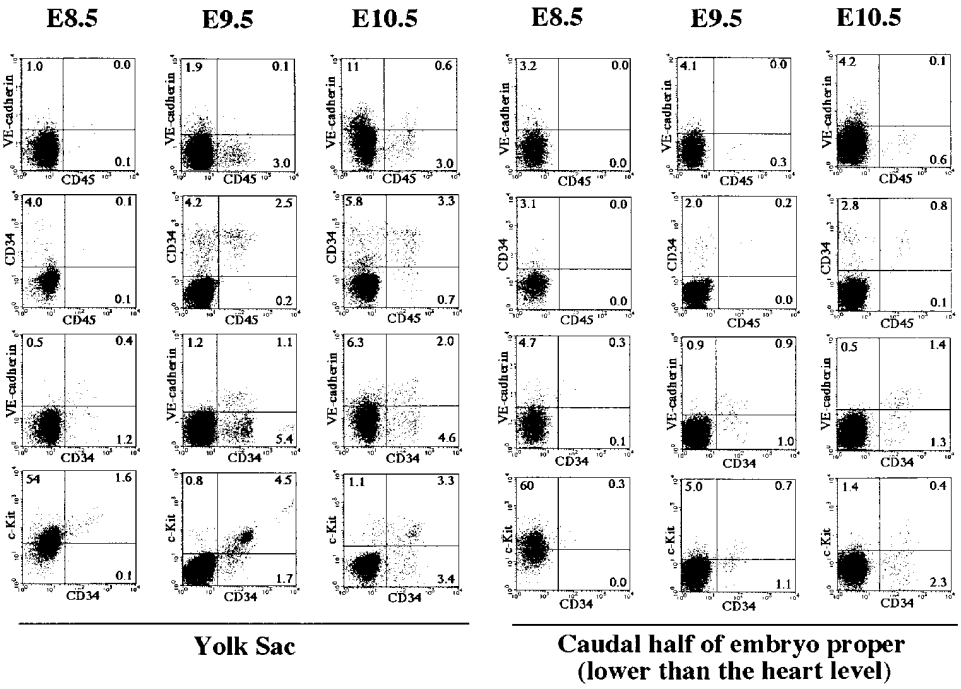

Figure 2. Surface Expression of VE-Cad, CD34, and CD45 in Embryos

YS and the caudal half of the embryo proper were dissected from E8.5, 9.5, and 10.5 embryos, and single cell suspensions were prepared. Cells were stained with mixtures of mAb conjugated to different fluorescence dyes and analyzed by FACS Vantage. Only living cells excluding propidium iodide were analyzed. Because of the variation in cell recoveries, the percentages presented in each panel may not represent the actual proportion of each subset in embryos. Note that all $CD45^+$ cells in early embryos are $CD34^+$, whereas almost none of the $CD45^+$ cells in E9.5 YS and the embryo proper are VE-cad^+. $CD45^+$ cells are detected earlier in YS than in the embryo proper. From E8.5 to E10.5, all VE-cad^+ cells coexpress CD34. Most cells dissociated from E8.5 embryo expressed low levels of c-Kit, while it is down-regulated within the next 24 hr.

and 4B). After making a thin film of the collagen gel, colonies were stained in situ by May-Grünwald-Giemsa to evaluate morphology of cells. In compact colonies, most cells were mononuclear (Figures 4C and 4D), whereas in diffuse colonies, either monocytes or polymorphonuclear cells with doughnut-shaped nuclei (Figures 4E and 4F) were found. This is the first demonstration of the existence of hematopoietic EC.

B Lymphopoietic Potential of the EC Fraction

After defining EC as VE-$cad^+CD45^-Ter119^-$, we next investigated the lymphohematogenic potential of EC and HPC sorted from YS and the caudal half of E8.5, 9.5, and 10.5 embryos. Lymphohematogenic potency was assessed in a culture containing OP9 stromal cells and IL-7 as described by Nakano et al. (1994).

EC from both YS and the caudal half of E8.5 embryos gave rise to $Gr1^+$ myelo-macrophage cell lineage (Figure 5A). $Ter119^+$ erythroid cells could also be detected in these cultures, when the cells were cultured in the presence of erythropoietin and analyzed on day 5 of the culture (data not shown). $B220^+$ cells were not detected in any cultures of cells from E8.5 embryos, be it from YS or the embryo proper. On the other hand, EC from both the caudal half and YS of E9.5 embryos grew extensively and gave rise to $B220^+$cells, most of which were IL-$7R\alpha^+$ and some already $sIgM^+$ (Figure 5B). Although a mixture of $CD45^+$ and $Ter119^+$ cells (Lin^+ cells) from the embryo proper generated $B220^+$ cells, those from YS did not. Hence, as far as E9.5 YS cells are concerned, lymphopoietic potential is enriched in the VE-cad^+ fraction rather than the Lin^+ fraction. Moreover, failure to generate $B220^+$ cells from the Lin^+ fraction indicated that the generation of B lineage cells from the EC fraction could not be due to contamination of HPC.

Since similar results were obtained in three independent experiments, including that presented in Figure 6, B lymphogenic HPC appears earlier in AGM than YS, though the activity of EC is the same between the two regions.

Presence of B lineage cells in cultures was also confirmed by room temperature polymerase chain reaction (RT-PCR) analysis of RAG1 and MB1 expression (Figure 6). Consistent with the results of FACS analysis, expression of RAG1 and MB1 in the culture of HPC from the YS was weak, whereas cultures of three other cell fractions were positive.

We also investigated whether or not our failure to detect B lymphogenic potential in the Lin^+ fraction of E9.5 YS is an outcome of suppressive activity of $Ter119^+$ erythroid cells on B lymphocyte differentiation, as they constitute the major population in the unsorted or Lin^+ populations of YS. In this particular experiment, $CD45^+$ rather than Lin^+ cells were purified and cultured under the same conditions. 80-fold fewer $B220^+$ cells were recovered

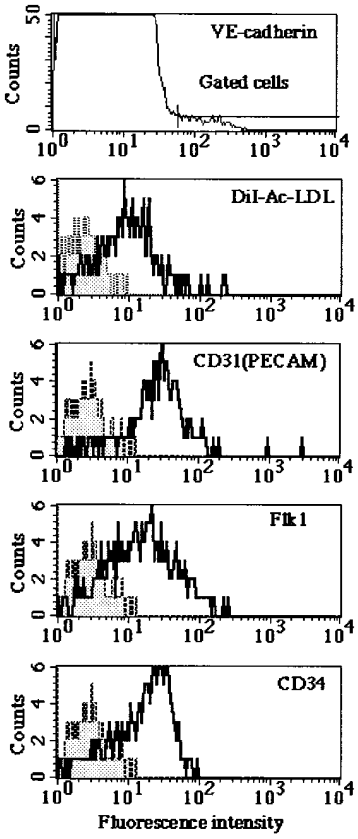

Figure 3. Expression of Vascular Markers in VE-Cad$^+$ Cells

YS and the caudal half of E9.5 embryos were dissected, pooled, and dissociated. The cells were incubated for 4 hr in the absence or presence of DiI-acetylated LDL (10 μg/ml) at 37°C and subsequently stained with APC-anti-VE-cad and FITC-anti-CD31, FITC-anti-Flk1, or FITC-anti-CD34 mAb. VE-cad$^+$ cells were gated, and expression of each marker was analyzed in the gated population. Dotted lines represent the controls for PE and FITC, without DiI-acetylated LDL incubation and with FITC-anti-mouse-IgG, respectively. Fluorescence of DiI was analyzed under the same condition for detecting PE. As shown in this figure, the entire VE-cad$^+$ cells shifted to the right after staining with CD31 or Flk1 or incubating with DiI-acetylated LDL. In contrast, by CD34 staining, a small proportion of cells did not shift to the right. These staining patterns indicate that almost all VE-cad$^+$ cells in E9.5 embryos coexpress CD31 and Flk1 and uptake-acetylated LDL, while a small proportion of them may not be positive in CD34 expression.

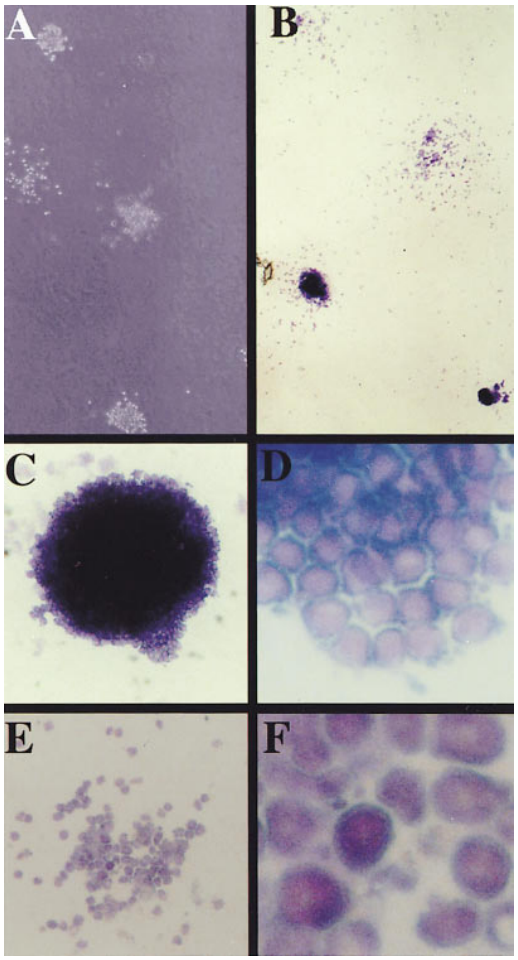

Figure 4. Colony Formation of VE-Cad$^+$ Cells in Response to Hematopoietic Growth Factors

YS and the caudal half of E9.5 embryos were dissected, pooled, and dissociated. After incubating them in the presence of DiI-acetylated LDL, the cells were stained with APC-anti-VE-cad and FITC-anti-CD31. The cells expressing all three markers were sorted and subjected to colony assay in type I collagen gel in the presence or absence of a mixture of SCF, IL-3, G-CSF, and erythropoietin. Each dish (3 cm) received 2 ml of medium containing 2500 cells. (A) After incubating 4 days, colonies of various shapes appeared. No colonies were generated in the absence of the growth factor mixture (data not shown). (B) Some dishes containing colonies were further stained by May-Grünwald-Giemsa solution. Both compact and diffuse colonies were generated. Compact colonies consist of round mononuclear cells (C), while polymorphnuclear cells and monocytes are found in diffuse colonies (E). Pictures of higher magnification (E and D) were made by computer processing with Adobe Photoshop software. Magnification: A and B, ×40; C and D, ×200; D and E, ×800.

from the culture of CD45$^+$ cells than from the culture of VE-cad$^+$ cells, indicating that B lymphogenic potency of YS cells is specifically enriched in the EC fraction (Figure 5C).

In E10.5 embryos, both EC and HPC from YS as well as the embryo proper generated B220$^+$ cells (Figure 5A).

T Lymphopoietic Potential of the EC Fraction

Results in the preceding section demonstrated B lymphopoietic potential of EC from E9.5 embryo. To elucidate whether or not EC of 9.5 embryos bear the potential to give rise to T cells, E8.5–10.0 embryos were fractionated into EC and HPC populations and subjected to thymic organ culture as described previously (Dou et al., 1995). Expecting that EC would not have the ability to home to intact thymic lobes, we cut dGuo-treated thymic lobes into four pieces, onto which EC or HPC were inoculated and incubated for 15 days. Thymic lobes were repaired spontaneously during culture. In each experiment, 10^4 unfractionated liver cells from E15 embryos were cultured under the same conditions. Before the appearance of hind limb-buds, HPC from neither YS nor the caudal half of the embryo proper could generate cells with the light scatter profile of lymphocytes (indicated by boxes). In contrast, EC from both regions

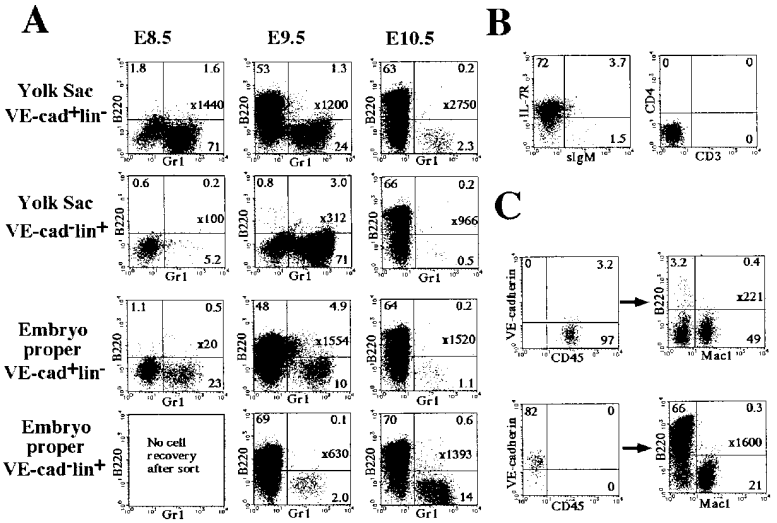

Figure 5. B Lymphopoietic Potential of EC in Embryos

(A) VE-cad$^+$Lin$^-$ and VE-cad-Lin$^+$ cells in YS and the caudal half of E8.5, 9.5, and 10.5 embryos were sorted, and 5000 cells were cultured with OP9 and IL-7. All cells were harvested, and round hemopoietic cells were enumerated, 14 days later. Cell increase rate against the input cell number was calculated and is presented in each panel. Harvested cells were stained with PE-anti-B220 and FITC-anti-Gr1. No B220$^+$ cells were observed in cultures of E8.5 embryos. In E9.5 embryos, defect of B cell genesis was found only in the culture of HPC fraction from YS.

(B) Expression of IL-7Rα, sIgM, CD3, and CD4 in the culture of EC fraction from E9.5 YS. Under this condition, no cells bearing the T cell markers are detectable.

(C) VE-cad$^+$ cells and CD45$^+$ cells were sorted from E9.5 YS and cultured with OP9 and IL-7. Culture cells were harvested, and the expression of B220 and Mac1 was analyzed with FACS Vantage, 15 days later. Cell increase rate against the input cell number is presented within each panel. B lymphopoietic progenitors are enriched in VE-cad$^+$CD45$^-$ population.

could give rise to cells in this lymphocyte gate, more than half of which were Thy1$^+$ (Figure 7, experiment 1). 4-fold more CD4/CD8 double and single positive cells were generated in the culture of EC from the caudal half of embryos than that from YS, indicating that the former contains more EC with T lymphopoietic potential than the latter.

Consistent with the results on B lymphopoietic potential, EC from E8.5 embryos could not give rise to T cells (Figure 7, experiment 2). When E10 embryos where hind limb-buds had just appeared were collected and subjected to the same assay, T lymphopoietic potential of EC was maintained in both regions. HPC from the caudal half of embryos but not that from YS could give rise to T cells, indicating that HPC with T lymphopoietic potential appear earlier in the embryo proper than YS (Figure 7, experiment 2). This result on T lymphopoietic potential is consistent with the result on B lymphopoietic potential (Figures 5 and 6) and confirms that HPC differentiated in the YS are not competent to give rise to lymphocytes, though EC in the same site is competent.

Discussion

Do Hematogenic EC Exist?

In this report, we demonstrated the lymphohematopoietic potential of VE-cad$^+$CD45$^-$Ter119$^-$ cells. If VE-cad expression is the hallmark of EC, our findings provide the first functional evidence for the existence of lymphohematopoietic EC. Thus, what should be discussed first is whether or not VE-cad$^+$Lin$^-$ cells represent EC. Although it is difficult to rule out the existence of non-EC VE-cad$^+$ cells, the following evidence suggests strongly that VE-cad$^+$ cells are indeed EC. First, our immunohistochemistry demonstrated that VE-cad is expressed exclusively in the EC of E9.5 YS, though VE-cad$^+$ cells do not form a complete EC layer in E7.5 embryos (Figure 1). Even though VE-cad$^+$ multipotent progenitors may exist in the early embryo, they should have differentiated to fully functional EC by E9.5. This conclusion is consistent with a previous study of Breier et al. (1996). Second, previous studies on the function of VE-cad indicated that VE-cad expression is sufficient for rendering the cells organized in the EC layer. Indeed, VE-cad is the sole cadherin involved in the establishment of adherence junction of EC (Lampugnani et al., 1995; Matsuyoshi et al., 1997; Vittet et al., 1997; Navarro et al., 1998). Moreover, it was shown that even a truncated form of VE-cad lacking β-catenin binding domain can function to mediate homotypic adhesion (Navarro et al., 1995, 1998). Third, VE-cad$^+$ cells in E9.5 embryos coexpress EC markers such as CD31, CD34, and Flk1 and take up acetylated LDL. In fact, our FACS analysis of embryos at various stages demonstrates that none of the presently available surface markers is more specific to EC than VE-cad (Figure 2 and our unpublished data). For example, CD31, CD34, and Flk1 are expressed in HPC as well as EC, as a proportion of CD45$^+$ cells express them. In contrast, the expression of VE-cad and CD45 is mutually exclusive. Moreover, a complete set of these four vascular markers is expressed only in VE-cad$^+$ cells. Taken together, VE-cad$^+$CD45$^-$Ter119$^-$ cells used in this study thus represent the fully functional EC that are integrated in the EC layer of the vascular system, particularly when they are sorted from E9.5 or E10.5 YS. Hence, our observation that VE-cad$^+$Lin$^-$ cells derived from embryos as late as E9.5–10.5 gave rise to lymphohematopoietic cell lineage is the first and unequivocal demonstration of the presence of lymphohematopoietic EC. This further suggests that histological pictures in which round cells are displayed as if they bud from the luminal wall of the vascular system (Smith and Glomski, 1982; Tavian et al., 1996) may indeed catch the process of HPC differentiation from the established EC layer. Such HPC budding

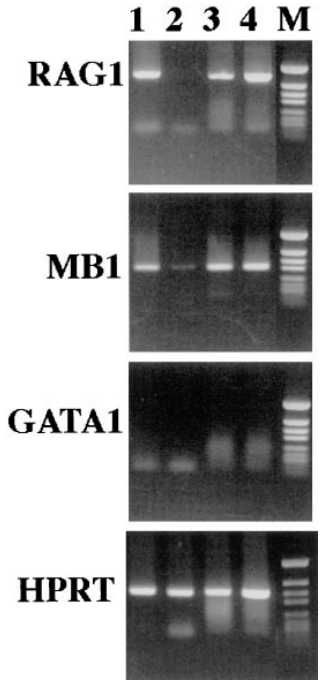

Figure 6. Expression of B Cell–Specific Genes in the Culture of EC

VE-cad$^+$Lin$^-$ and VE-cad$^-$Lin$^+$ cells were enriched from E9.5 YS and the caudal half of the embryo proper, and 5000 cells were cultured for 14 days with OP9 and IL-7. Cultured cells were harvested and total RNA was prepared for RT-PCR analysis of the expression of RAG1, MB1, GATA1, and HPRT. Lane 1, VE-cad$^+$Lin$^-$ YS cells; lane 2, VE-cad$^-$Lin$^+$ YS cells; lane 3, VE-cad$^+$Lin$^-$ cells from the embryo proper; lane 4, VE-cad$^-$Lin$^+$ cells from the embryo proper; and lane M, a size marker.

has been demonstrated only in large vessels in the AGM region. However, we showed that more than 10% of VE-cad$^+$CD31$^+$ cells from E9.5 embryo generated colonies in response to the mixture of hematopoietic factors. Moreover, VE-cad$^+$ cells from YS can give rise to HPC. All of these findings indicate that HPC differentiation from the EC layer is a common event in the early sites of blood cell differentiation, be it YS or the embryo proper.

Diversity of Lymphohematopoietic Potential among Newly Generated EC

Here, we reported a situation in which cells with lymphopoietic potential were enriched in EC rather than HPC. In both YS and the caudal half of E9.5 embryos before hind limb-buds appear, cells that are competent to give rise to T lymphocytes are enriched in the VE-cad$^+$CD45$^-$ rather than VE-cad$^-$CD45$^+$ fraction. Thus, the competency to generate lymphocytes appears earlier in EC than HPC.

Our results also indicate that EC become competent to differentiate into lymphocytes with a 1-day time lag following the acquisition of hematopoietic potential. This failure to generate lymphocytes from EC of E8.5 embryos may be due to a deficiency of our culture conditions for supporting the lymphoid differentiation of earlier progenitors. However, Nakano et al. (1994) showed that a combination of OP9 feeder cells and IL-7 supports B cell generation from the totipotent ES cells. We also demonstrated that this culture condition is effective in inducing B cells from freshly isolated embryonic ectoderm and mesoderm (Kanatsu and Nishikawa, 1996). Hence, this failure likely reflects the intrinsic potential of EC present in E8.5 embryo rather than the culture conditions. Thus, acquisition of lymphopoietic competency may be an independent event distinct from that of hematopoietic competency during EC differentiation. Likewise, EC generated in nonhematopoietic sites such as the cranial region cannot give rise to hematopoietic cells at all (our unpublished data), indicating that a diverse set of EC is generated during early embryogenesis. With respect to lymphohematopoietic potential, at least three cell types may exist: nonhematopoietic, hematopoietic without lymphopoietic competency, and lymphohematopoietic EC. If environmental cues play a role in determining the proportion of each subset, only mesoderm that differentiates in the right time and place can generate EC with multiple potentials. Recently, Eichmann et al. (1997) demonstrated that vascular endothelial growth factor suppressed in vitro differentiation of Flk1$^+$ cells to HPC. If the concentration of vascular endothelial growth factor differs significantly among embryonic regions, this could be a molecular mechanism underlying the environmental regulation of fate determination during mesoderm differentiation to EC. Further study is needed to understand the molecular nature of the environmental cues for generating the diversity of EC.

Origin of HPC with Lymphopoietic Potential

Our data indicate that YS and the embryo proper do not differ in the lymphopoietic potential of EC, though somewhat higher activity to generate T cells is detected in the embryo proper. Paradoxically, we also found an embryonic stage when CD45$^+$ HPC in the embryo proper but not in YS are competent to give rise to lymphocytes. If acquisition of hematopoietic and lymphopoietic competencies requires distinct processes, an interpretation of this paradoxical observation is that YS does not provide an effective environment for the acquisition of lymphopoietic competency during differentiation from EC to HPC. Alternatively, lymphopoietic HPC may not be able to survive in the YS microenvironment, though the presence of lymphopoietic HPC in E10.5 YS, be it generated in situ or migrated from the embryo proper, suggests that this possibility is unlikely. It is also possible that YS is not the site of HPC generation from EC, although they have the potential to give rise to lymphocytes under our culture conditions. If so, HPC in the YS should be the progenies derived from the earliest wave of hematopoiesis from mesoderm or hemangioblast or both during blood island formation, whereas those in the embryo proper are from EC. This model may be more consistent with the previous model that hypothesizes that the primitive hematopoiesis is generated in YS from hemangioblasts, whereas the definitive hematopoietic cells bud from the EC layer of the AGM (Dzierzak and Medvinsky, 1995; Dieterlen-Lievre et al., 1997). In either model, differentiation from EC to HPC should be regulated distinctively in the embryo proper and YS, while that from mesoderm to EC occurs in a similar way in the two regions. This conclusion supports the notion

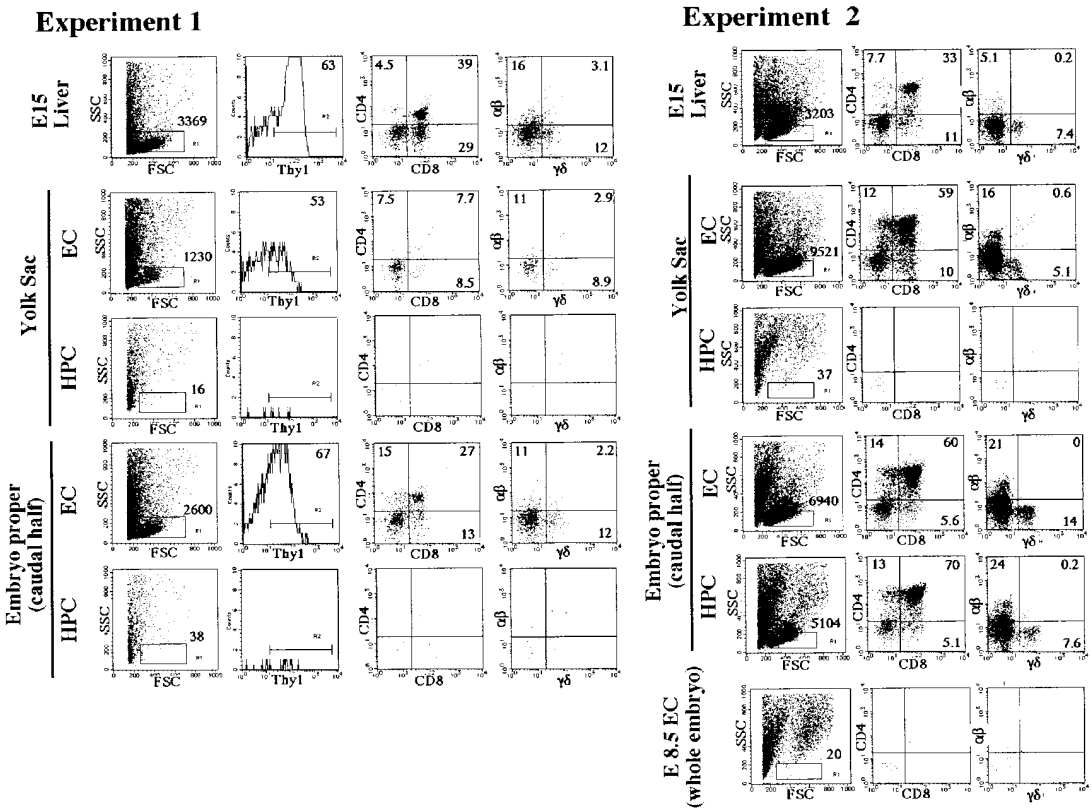

Figure 7. T Lymphopoietic Potential of EC in Embryos

(Experiment 1) VE-cad$^+$Lin$^-$ (EC) and VE-cad$^-$Lin$^+$ (HPC) fractions were sorted from YS and the caudal half of E9.5 embryos before appearance of hind limb-buds. 300 EC or 1000 VE-cad$^-$Lin$^+$ HPC cells were inoculated onto dGuo-pretreated thymus lobes and incubated for 15 days. Triplicate culture was set for each group and pooled at the time of assay. Numbers presented in each light scatter figure (the first column) represent the event count detected in the lymphocyte gate indicated by the square. Cells in this gate were analyzed for Thy1 expression (the second column). Expression levels of CD8/CD4 (the third column) and $\gamma\delta/\alpha\beta$ T cell receptors (the fourth column) in Thy1$^+$ cells in the lymphocyte gate are presented. As a positive control, 10^4 unfractionated fetal liver cells from E15 embryo were cultured under the same condition.
(Experiment 2) E8.5 embryos and E10 embryos with hind limb-buds were used for the assay. VE-cad$^+$CD45$^-$ (EC) and VE-cad$^-$CD45$^-$ (HPC) cells were sorted from various portions of embryos and subjected to thymus organ culture. In this experiment, 1000 cells were inoculated to each culture. After 15 days, thymus lobes of each group were pooled and analyzed for expression of either CD4/CD8 or $\alpha\beta/\gamma\delta$. The number of events counted in the lymphocyte gate was presented in the panels of the light scatter profile. The culture of 10^4 unfractionated fetal liver cells from E15 embryos were used as a positive control. No T lymphocytes are detected in the culture of EC from E8.5 embryos. In the E10 embryos, all fractions but HPC from YS can generate T cells.

that YS and the embryo proper differ in their ability to generate HPC with lymphopoietic potential (Ogawa et al., 1988; Muller et al., 1994; Dzierzak and Medvinsky, 1995; Cumano et al., 1996; Medvinsky and Dzierzak, 1996). It should be noted, however, that our observation is also compatible with reports arguing against the AGM origin of the definitive hematopoiesis. Recently, Yoder et al. (1997) demonstrated that CD34$^+$c-Kit$^+$ cells from E9.0 YS as well as AGM can give rise to lymphocytes upon transfer to bone marrow–ablated neonatal mice. Although they claimed that CD34$^+$c-Kit$^+$ cells represent HPC, our observation demonstrated clearly that CD34$^+$ c-Kit$^+$ cells contain both CD45$^+$ and VE-cad$^+$ fractions (Figure 2). If the cell preparation used in the studies by Yoder et al. (1997) was contaminated with EC fraction, and if their experimental conditions are sufficient to support EC differentiation to lymphocytes, it is conceivable that they were able to generate lymphocytes from YS cells. Yoder et al. (1997) emphasized the importance of using neonatal rather than adult mice as the hosts for inducing lymphocytes from YS cells. Such a strict requirement of the microenvironment for the differentiation to lymphocytes might reflect the property of EC rather than HPC.

With respect to the problem of embryonic circulation that may contribute to the interchange of cells with lymphopoietic potency between YS and the embryo proper, Cumano et al. (1996) performed an elegant tissue explant experiment to show that YS has no potential to generate HPC with lymphopoietic potential. We agree with Cumano et al. (1996) and also with Medvinsky and Dzierzak (1996) in that YS is fated not to generate HPC with lymphopoietic potential before E10. However, we also showed that EC in YS do have lymphopoietic potential. Thus, we want to emphasize that the issue to be addressed in the future is the molecular mechanisms that fate lymphopoietic EC in YS incapable of generating HPC with lymphopoietic competency, rather than interchange of HPC between two regions.

The origin of HPC with lymphogenic potential is an

issue dealing with developing tissues whose cellular composition is changing dynamically. In such tissues, the potential and fate of each cellular component may often be dissociated, thereby rendering the fate analysis difficult. We believe that such a situation can be analyzed properly by evaluating both fate and potential of each individual component. Here, we present an example showing that VE-cad$^+$ and CD45$^+$ components in the YS display distinctive lymphohematopoietic potentials, thereby providing a clue as to why mutually conflicting results have been obtained. Moreover, our observation strongly suggests that fate determination during differentiation from mesoderm, EC, and HPC is determined by environmental components developing in parallel. This is demonstrated also in the recent study of Turpen et al. (1997) in which different regions with hematopoietic potential were transplanted in various sites of frog embryos. While the transplantation of embryonic tissues is difficult to perform in the murine embryo, the method to separate intermediate stages during differentiation from multipotent mesoderm to committed HPC can be utilized to study the molecular mechanisms underlying environmental regulation of the commitment to HPC with lymphogenic potential.

Experimental Procedures

Cell Preparation

Embryos were obtained and staged as previously described (Kanatsu and Nishikawa, 1996; Takakura et al., 1997). In this study, E8.5, E9.5, and E10 stand for embryos with 5–10 somites, 20–30 somites and fore limb-buds only, and both fore and hind limb-buds, respectively. For dissection, YS was removed from the embryo proper, and the remaining embryo was cut in half at the level below the heart. The caudal half of this prepared embryo was used as the embryo proper. Dissected YS and the caudal half of embryos were first incubated with dispase II solution (2.4 U/ml) (Boehringer Mannheim, Germany) in phosphate-buffered saline, washed once, and subsequently incubated with cell dissociation buffer containing EDTA/EGTA (Cat. No. #13150–016, GIBCO-BRL, Rockville, MD). Cells were dissociated by gentle pipetting in this solution and then washed with phosphate-buffered saline containing 5% fetal calf serum. Large cell clumps were removed with a nylon mesh.

Antibodies

Anti-VE-cad (VECD1) (Matsuyoshi et al., 1997), anti-Flk1 (AVAS12) (Kataoka et al., 1997), anti-IL-7Rα (Sudo et al., 1993) and anti-c-Kit (ACK2) (Nishikawa et al., 1991) mAb were prepared and conjugated to either biotin, R-phycoerythrin (PE), or FITC (all from Molecular Probes, Eugene, Oregon) as described previously (Nishikawa et al., 1991; Ogawa et al., 1991). Unconjugated, FITC-conjugated, or PE-conjugated anti-B220, anti-Gr1, anti-IgM, anti-CD31, anti-CD34, anti-CD4, anti-CD8, anti-$\gamma\delta$, anti-$\alpha\beta$ mAb, and apophycocyanin (APC)-conjugated anti-Thy1 mAb were purchased from Pharmingen (San Diego, CA).

Surface Staining, Cell Sorting, and Immunohistochemistry

For surface staining, cell suspensions were incubated on ice in the presence of various mixtures of labeled mAb. When biotin-conjugated mAb were used, they were washed and incubated with streptavidin conjugated to either PE or APC. Labeled cells were resuspended in Hank's balanced salt solution containing 1% bovine serum albumin and propidium iodide and analyzed with FACS Vantage (Becton Dickinson, San Jose, CA). Dead cells stained with propidium iodide were excluded. Cell sorting was performed as described previously (Ogawa et al., 1991). In some experiments, dissociated cells were first incubated with 10 μg/ml DiI-acetylated LDL (Molecular Probes) for 4 hr and then stained with mixtures of mAb. Fluorescence of DiI was detected under the same conditions used for PE. The procedure for whole-mount immunohistostaining was described previously (Takakura et al., 1997).

Culture with OP9 Stromal Cell Line

For generating erythroid cells, 5000 sorted cells were cultured with OP9 stromal cell line with αMEM medium (GIBCO) containing 5×10^{-5} M 2-mercaptoethanol, 2 U/ml erythropoietin (a gift from Kirin Co. Ltd) and 100 ng/ml SCF (Kodama et al., 1994; Nakano et al., 1994). Erythroid precursors expressing Ter119 were evaluated by FACS Vantage 5 days after the culture. Assay for B and myeloid cell lineage was performed by culturing 5000 cells with OP9 and 200 U/ml IL-7. Triplicate cultures were set for each assay. Cells were harvested, pooled, counted, and analyzed for the proportions of B220$^+$ and Gr1$^+$ or Mac1$^+$ cells 14 to 15 days later. In some experiments, surface expression of IL-7Rα and IgM was assessed.

RT-PCR Analysis

Total cellular RNA was isolated from cultured cells using Isogen (Nippon Gene, Japan). First strand cDNA was prepared by reverse transcription according to the protocol provided by the manufacturer (Perkin Elmer Cetus, Norwalk, CT). Oligo (dT)-primed cDNA was prepared from total RNA derived from 2×10^5 cells using avian myeloblastosis virus reverse transcriptase in a reaction volume of 20 μl under conditions recommended by the manufacturer (GIBCO-BRL). PCR conditions were optimized for each primer set to maintain amplification in the linear range. Primer pairs were as follows: RAG1, 5′ sequence (5′) 5-TGCAGACATTCTAGCACTCTGG-3 and 3′ sequence (3′) 5-ACATCTGCCTTCACGTCGAT-3; Mb1, (5′) 5-GCCAG GGGGTCTAGAAGC-3 and (3′) 5-ACATCTGCCTTCACGTCGAT-3; GATA1, (5′) 5-AGTGCTGGGATTACAGGCAT-3 and (3′) 5-TCATGGT GGTAGCTGGTAGC-3; and HPRT (5′) 5-GAGCTACTGTAATGATCAG TCAACGG-3 and (3′) 5-GATTCAACTTGCGCTCATCTTAGGC.

High-Oxygen Thymic Organ Culture

T lymphogenic potency was assessed by using high-oxygen thymus organ culture as described previously (Dou et al., 1995). EC (300) or HPC (1000) were cultured in each dGuo (Sigma)-treated thymic lobe in the presence of 100 ng/ml SCF. Before cell inoculation, thymus lobes were cut into four pieces to facilitate the migration of cells that may not be able to migrate through the capsule. These cuts were cured spontaneously during incubation. Triplicate cultures were set for each assay. Lobes (three) were harvested, pooled, and split into two aliquots 15 days later. Each aliquot was two-color stained with CD8(FITC)/CD4(PE) or $\gamma\delta$(FITC)/$\alpha\beta$(PE) or three-color stained with CD8(FITC)/CD4(PE)/Thy1(APC) or $\gamma\delta$(FITC)/$\alpha\beta$(PE)/Thy1(APC) mAb mixtures and analyzed using FACS Vantage. Only cells with the light scatter profile of lymphocytes were counted and analyzed. In each experiment, 10^4 liver cells from E15 embryos were cultured as a positive control.

Colony Formation of Hematopoietic Cells in Type I Collagen Gel

α-MEM containing 0.09% type I collagen was used as a semisolid medium for colony assay (Kodama et al., 1994). For colony assay, 2 ml of medium containing 2500 cells was placed in a 3 cm dish that was kept on ice. After addition of a growth factor mixture containing 100 ng/ml SCF, 200 U/ml IL-3, 100 ng/ml G-CSF, and 2 U/ml erythropoietin, the dish was transferred to 37°C to polymerize collagen. Colonies were counted by inverted microscope 4 days after the initiation of culture. To perform cytological analysis, the medium in the dish was blotted off by blotting papers through a sheet of cellulose membrane (Clean Wipe-P, Asahi Kasei Co. Ltd, Japan) placed on the surface of the gel. This thin film of collagen gel–containing colonies was dried and subjected to cytological analysis with May-Grünwald-Giemsa staining.

Acknowledgments

We thank N. Matsuyoshi for VECD1 mAb and L. Zon and S. Fraser for critical reading of the manuscript. This study is supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan (No. 07CE2005, 06277102, 06NP1101).

References

Ayalon, O., Sabanai, H., Lampugnani, M.G., Dejana, E., and Geiger, B. (1994). Spatial and temporal relationships between cadherins and PECAM-1 in cell-cell junctions of human endothelial cells. J. Cell Biol. *126*, 247–258.

Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnurch, H., Gotsch, U., Vesweber, D., Risau, W., and Dejana, E. (1996). Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. Blood *87*, 630–641.

Cumano, A., Dieterlen-Livere, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell *86*, 907–916.

Dieterlen-Lievre, F., Godin, I., and Pardanaud, L. (1997). Where do hematopoietic stem cells come from? Int. Arch. Allergy Appl. Immunol. *112*, 3–8.

Dou, Y.M., Watanabe, Y., Aiba, Y., Wada, K., and Katsura, Y. (1995). A novel culture system for induction of T cell development: modification of fetal thymus organ culture. Thymus *23*, 195–207.

Dzierzak, E., and Medvinsky, A. (1995). Mouse embryonic hematopoiesis. Trends Genet. *11*, 356–366.

Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Brant, C., and Le Douarin, N. (1997). Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. Proc. Natl. Acad. Sci. USA *94*, 5141–5146.

Johnson, G., and Moore, M. (1975). Role of stem cell migration in initiation of mouse foetal liver hemopoiesis. Nature *258*, 726–729.

Kanatsu, M., and Nishikawa, S.I. (1996). In vitro analysis of epiblast tissue potency for hematopoietic cell differentiation. Development *122*, 831–838.

Kataoka, H., Takakura, N., Nishikawa, S., Tsuchida, K., Kodama, H., Kunisada, T., Risau, W., Kita, T. and Nishikawa, S.I. (1997). Expression of PDGF receptor, c-Kit and FLK1 gene clustering in mouse chromosome 5 define distinct subsets of nascent mesoderm cells. Dev. Growth Differentiation *39*, 729–740.

Kodama, H., Nose, M., Niida, S., Nishikawa, S., and Nishikawa, S.I. (1994). Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. Exp. Hematol. *22*, 979–984.

Lampugnani, M.G., Caveda, L., Breviario, F., Del-Maschio, A., and Dejana, E. (1993). Endothelial cell-to-cell junctions. Structural characteristics and functional role in the regulation of vascular permeability and leukocyte extravasation. Baillieres Clin. Haematol. *6*, 539–558.

Lampugnani, M.G., Corada, M., Caveda, L., Breviario, F., Ayalon, O., Geiger, B., and Dejana, E. (1995). The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). J. Cell Biol. *126*, 247–258.

Matsuyoshi, N., Toda, K., Horiguchi, Y., Tanaka, T., Nakagawa. S., Takeichi, M. and Imamura, S. (1997). In vivo evidence of the critical role of cadherin-5 in murine vascular integrity. Proc. Assoc. Am. Physicians *109*, 362–371.

Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. Cell *86*, 896–906.

Moore, M., and Owen, J. (1967). Stem cell migration in developing myeloid and lymphoid systems. Lancet *11*, 658–659.

Muller, A., Medvinsky, A., Strouboulis, J.Grosveld, F., and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. Immunity *1*, 291–301.

Nakano, T., Kodama, H., and Honjo, T. (1994). Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science *265*, 1098–1101.

Navarro, P., Caveda, L., Breviario, F., Mandoteanu, I., Lampugnani, M.G., and Dejana, E. (1995). Catenin-dependent and -independent functions of vascular endothelial cadherin. J. Biol. Chem. *270*, 30965–30972.

Navarro, P., Ruco, L., and Dejana, E. (1998). Differential localization of VE- and N-cadherins in human endothelial cells: VE-cadherin competes with N-cadherin for junctional localization. J. Cell Biol. *140*, 1475–1484.

Nishikawa, S., Kusakabe, M., Yoshinaga, K., Ogawa, M., Hayashi, S., Kunisada, T., Era, T., Sakakura, T., and Nishikawa, S.I. (1991). In utero manipulation of coat-color formation by a monoclonal c-Kit antibody: two distinct waves of c-kit-dependency during melanocyte development. EMBO J. *10*, 2111–2118.

Nishikawa, S.I., Nishikawa, S., Hirashima, M., Matsuyoshi, N., and Kodama, H. (1998). Progressive lineage analysis by cell sorting and culture identifies $Flk1^{+}VE\text{-}cadherin^{+}$ cells at a diverging point of endothelial and hemopoietic lineages. Development *125*, 1747–1757.

Ogawa, M., Nishikawa, S., Ikuta, K., Yamamura, F., Naito, M., Takahashi, K., and Nishikawa, S.I. (1988). B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. EMBO J. *7*, 1337–1343.

Ogawa, M., Matsuzaki , Y., Nishikawa, S., Hayashi, S.I., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H., and Nishikawa, S.I. (1991). Expression and function of *c-kit* in hemopoietic progenitor cells. J. Exp. Med. *174*, 63–71.

Smith, R.A., and Glomski, C.A. (1982). "Hemogenic endothelium" of the embryonic aorta: does it exist? Dev. Comp. Immunol. *6*, 359–368.

Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M., Yoshida, H., and Nishikawa, S.I. (1993). Expression and function of the interleukin-7 receptor in murine lymphocytes. Proc. Natl. Acad. Sci. USA *90*, 9125–9129.

Takakura, N., Yoshida, H., Ogura, Y., Kataoka, H., Nishikawa, S., and Nishikawa, S.I. (1997). PDGFRa expression during mouse embryogenesis: immunolocalization analyzed by whole mount immunostaining using the monoclonal anti-mouse-PDGFRa antibody. J. Histochem. Cytochem. *45*, 883–892.

Tavian, M., Coulombel, L., Luton, D., Clemente, H.S., Dieterlen-Lievre, F., and Peault, B. (1996). Aorta-associated $CD34^{+}$ hematopoietic cells in the early human embryo. Blood *87*, 67–72.

Turpen, J.B., Kelley, C.M., Mead, P.E., and Zon, L.I. (1997). Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo. Immunity *7*, 325–334.

Vittet, D., Buchou, T., Schweitzeritzer, A., Dejana, E., and Huber, P. (1997). Targeted null mutation in the vascular endothelial-cadherin gene impaires the organization of vascular like strucutures in embryoid bodies. Proc. Natl. Acad. Sci. USA *94*, 6273–6278.

Weissman, I.L., Papaioannou, V., and Gardner, R. (1978). Fetal hematopoietic origins of the adult hematolymphoid system. In Cold Spring Harbor Conferences on Cell Proliferation, Volume 5, Differentiation of Normal and Neoplastic Hematopoietic Cells, P.A. Mark and J.E. Till, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp 33–47.

Yoder, M.C., Hiatt, K., and Mukherjee, P. (1997a). In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. Proc. Natl. Acad. Sci. USA *94*, 6776–6780.

Yoder, M.C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D.M., and Orlic, D. (1997b). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. Immunity *7*, 335–344.